FACTOR ANALYSIS OF FERMENTATION FOR THE PRODUCTION OF BIOPOLYMER IN SHAKE-FLASKS

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"I hereby declare that I had read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the purpose of the granting of Bachelor of Chemical Engineering with Biotechnology."

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Date : ……

I declare that this thesis entitled *"Factor Analysis of Fermentation for the Production of Biopolymer in Shake-Flasks"* is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree

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Name of candidate : Nafsiah binti Hussain

Date : ……

“Words and tears
can never express the love
and sacrifice you all have
given by enlisting”.

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ABSTRACT

Poly(β- hydroxybutyric acid) or (PHB) is a natural, biodegradable polymer, which is accumulated as an energy reserve material by a large number of bacteria when nutrient such as nitrogen source is available in limiting concentrations in the present of excess carbon source. The major problem associated with the industrial production of PHB is its high production cost. In the present study, effort was made to screen out the factors of the experiment that influence the production of biomass that is *Cupriavidus necator* and also PHB. The factors was including culture media such as glucose, $CaCl_2.2H_2O$, trace element, $(NH_4)_2SO_4$ and $Na_2HPO_4.7H_2O$ agitation and temperature. Matlab and F-Test distribution have been use to screen out insignificant factor. These factors can then be classified into categories according to how significantly each of them affects the yield. If experimental variable contribute only to factors do not affect the yield significantly then it can be concluded that it is not relevant to the yield and can be dropped from subsequent experiments. If an experimental variable contributes to one or more factors which have significant effects on the yield then the experimental variables is relevant to the yield and should be retained for future investigation an optimization.

ABSTRAK

Poly(β- hidroksibutric asid) (PHB) adalah polimer yang mempunyai sifat mudah terurai dengan adanya aktiviti mikroorganisma (*Cupriavidus necator*). PHB berperanan sebagai pembekal tenaga kepada mikrooganisma apabila nutrin seperti nitrogen berada dalam keadaan terhad manakala kehadiran karbon dalam keadaan berlebihan. Faktor utama yang menghadkan pengeluaran PHB dalam industri ialah kos untuk menghasilkan PHB terlalu tinggi berbanding dengan polimer berasakan petrokimia.. Oleh sebab itu, banyak kajian yang telah dibuat untuk mengurangkan kos penghasilan biopolimer ini. Dalam kajian ini, analisis faktor telah digunakan untuk menapis pembolehubah-pembolehubah seperti kultur media dan antaranya adalah gula, $CaCl_2.2H_2O$, unsur penyurih, $(NH_4)_2SO_4$ dan $Na_2HPO_4.7H_2O$ serta suhu dan kadar goncang.. Kaedah matlab dan taburan F-Test telah digunakan untuk menapis pembolehubah- pembolehubah yang telah disebutkan. Kemudian, pembolehubah-pembolehubah boleh dikelaskan kepada katogeri berdasarkan bagaimana pembolehubah- pembolehubah tersebut mempengaruhi hasil. Jika pembolehubah tidak mempengaruhi penghasilan PHB, pembolehubah tersebut boleh diasingkan daripada eksperimen manakala sekiranya pembolehubah mempengaruhi penghasilan PHB, pembolehubah tersebut boleh dikekalkan untuk proses seterusnya iaitu optimasi

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LIST OF SYMBOLS

R.P.M	-	Shaking speed
T	-	Temperature
G	-	Glucose
P	-	Na_2HPO_4
A	-	$(NH_4)_2SO_4$
C	-	CaCl2
TE	-	Trace element
F	-	Factor
MSE	-	Mean Square error
$F_{0.95}$	-	F-Test distribution
d.f	-	Degree of freedom

CHAPTER 1

INTRODUCTION

1.1 Introduction

Poly(β- hydroxybutyric acid) (PHB) is thermoplastic that widely produced by many bacteria such as *Protomonas extroquens*, *Candida utilis* ATCC 8205, *Azetobacter vinelandii* and *Cupriavidus necator* which is accumulated in the form of intracellular granules. This granules act as energy reserve materials when nutrient such as nitrogen and phosphorus sources are available in limiting concentrations in the present of excess carbon source. However, this research area is mainly associated with *Cupriavidus necator,* since it accumulate PHB more than any other wild type microbes [1]. PHB is a biodegradable, biocompatible thermoplastic and similar physical properties to polypropylene. The use of PHB as biodegradable plastic is desirable since the non-biodegradable plastics disposal, after they are used, causes significant ecological problems. That is, the availability of landfills is limited and the incineration of plastic increases greenhouse gases and releases toxic compounds [2].

The method of Factor Analysis [3, 4, 5] have been used to screen the experimental variables which are most relevant to the fermentation. This method has been shown to allow an efficient screening of the experimental variables which are most relevant to the biomass yield in a shake flask.

The method of Factor Analysis [3, 4, 5] enables us to describe the various experimental variables in term of mutually orthogonal factors which are uncorrelated to each other but which have the same mean and the same variance as the standardized from the experimental variables. Mutually orthogonal factors are important in that only such factors may be use to construct linear models, where the interactions between factors are not taken into account. Empirical models are constructed to describe the yields in term of mutually orthogonal factors. The significance of each factor in its effect on the yield is the determined by removing the particular factor from the model involving all the factors and comparing the mean square difference between the actual data and the prediction of the resulting model with the mean square difference between the actual data and the predictions of the model involving all the factors using the F-test.

These factors can then be classified into categories according to how significantly each of them affects the yield. If experimental variable contribute only to factors do not affect the yield significantly then it can be concluded that it is not relevant to the yield and can be dropped from subsequent experiments. If an experimental variable contributes to one or more factors which have significant effects on the yield then the experimental variables is relevant to the yield and should be retained for future investigation an optimization.

1.2 Problem statement

Currently the main problem, which limits the widespread use of PHB and its copolymers, is its relatively high cost compared with plastics based on petrochemicals. One of the major factors adding to the cost of PHB is the cost in product recovery in fermentation process and substrates used for production. Several studies have tackled this problem using different approaches. Some researcher have focus on reducing cost by optimizing fermentation processes of *Cupriavidus necator*, expressing the operon responsible for PHB production in other organism such as *Escherichia coli* [2]. One

approached to minimize the consumption of glucose in order to optimize the usage. Therefore, less expensive substrates, improved cultivation strategies and easier downstream processing methods are required for reducing the cost. Thus, utilization of media containing cheaper carbon and nitrogen sources should be used to reduce the production cost of PHB [6]

1.3 Objective of study

. The objective of this study is to determine which factors influence the most in production of biomass and PHB.

1.4 Scopes of study

The scopes of this research are to study the effect of variables such as culture media (glucose, $CaCl_2.2H_2O$, trace element, $(NH_4)_2SO_4$ and $Na_2HPO_4.7H_2O$) agitation and temperature in the production of biomass and PHB using matlab and F-Test.

CHAPTER 2

LITERATURE REVIEW

2.1 Poly β hydroxybutyric (PHB)

Poly β hydroxybutyric (PHB) is polyesters produced by a wide range of bacteria when they find themselves in an environment with an available carbon source but limited in additional nutrient(s) required for growth. The short-chain-length PHAs, where R is a methyl or ethyl, have properties of thermoplastics and are biodegradable (Figure 2.1).

PHAs are synthesized by many living organisms. The main candidates for the large-scale production of PHAs are plants and bacteria. Plant cells can only cope with low yields 10% w/w of dry weight of PHA production. High levels 10–40% w/w of dry weight of polymer inside the plant have a negative effect on the growth and development of the plant. At present, this problem has not been overcome [7]. In contrast, within bacteria, PHAs are accumulated to levels as high as 90% w/w of the dry cell mass [8]. Accumulating PHAs is a natural way for bacteria to store carbon and energy, when nutrient supplies are imbalanced. These polyesters are accumulated when bacterial growth is limited by depletion of nitrogen, phosphorous [9] or oxygen and an excess amount of a carbon source is still present. While the most common limitation is nitrogen, for some bacteria, such as *Azotobacter* spp. the most effective limitation is oxygen [10].

As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells. It is advantageous for bacteria to store excess nutrients inside their cells, especially as their general physiological fitness is not affected. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state. Thus, leakage of these valuable compounds out of the cell is prevented and the nutrient stores will remain securely available at a low maintenance cost [11].

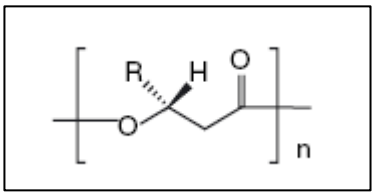

Figure 2.1: Poly (3-hydroxyalkanoates)

2.2 Chemical structure of PHA's

Besides PHB, there are many other PHAs composed of 3- hydroxy fatty acids. The pendant group (R) varies from methyl (C_1) to tridecyl (C_{13}). Fatty acids with the hydroxy group at position 4, 5 or 6 and pendant groups containing substituents or instaurations are also known. Within bacterial metabolism, carbon substrates are converted into hydroxyacyl-CoA thioesters. As shown in Figure 2.2, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. This polymerization reaction is catalysed by the host's PHA synthase.

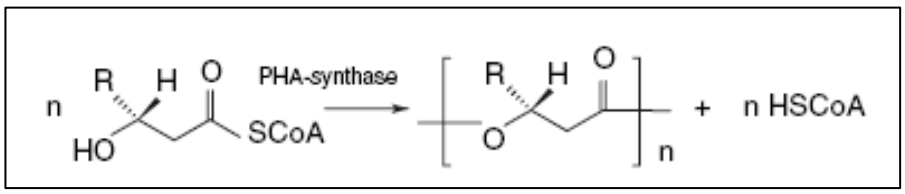

Figure 2.2: Synthesis of PHAs in bacteria hydroxyacyl-CoA thioesters as precursor.

In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the stereochemica (R)-configuration. There is an enormous variation possible in the length and composition of the side chains. This variation makes the PHA polymer family suitable for an array of potential applications. The most common are shown in Table 2.1.

Table 2.1: PHAs and corresponding R-groups

R-Group	Full name	Short
CH_3	Poly (3-hydroxybutyrate)	PHB
CH_2CH_3	Poly (3-hydroxyvalerate)	PHV
$CH_2CH_2CH_3$	Poly (3-hydroxyhexanoate)	PHHx

2.4 Physical properties

The material characteristics of these biopolymers are similar to conventional plastics such as polypropylene [12]. The properties of PHB (homopolymer), PHBV, PHB4B (scl-copolymers) and PHBHx (mcl-copolymer) are compared with polypropylene (PP) in Table 2.1. PHB homopolymer is a highly crystalline [13], stiff but brittle material. When spun into fibres it behaves as a hard-elastic material [14]. Copolymers like PHBV or mcl-PHAs are less stiff and brittle than PHB, while retaining most of the other mechanical properties of PHB. Homopolymer PHB has a helical crystalline structure, this structure seems to be similar in various copolymers. Melting behavior and crystallization of PHAs have recently been studied by Gunaratne and Shanks (2005). In this study, PHAs show multiple melting peak behaviour and melting–recrystallization–remelting. When processing biopolymers, it is important to know the point of thermal degradation. Carrasco et al. (2006) recently determined that PHB (Biopol) decomposition starts at 246.3^{o}C, while the value for PHBV (Biopol) is 260.4^{o}C. This indicates that the presence of valerate in the chain increases the thermal stability of the polymer

Table 2: Properties of PHAs and polypropylene (PP). PHBV contains 20% 3HV monomers, PHB4B) contains 16% 4HB-monomers, PHBHx contains 10% 3HHx monomers (Tsuge 2002)

Parameter	PHB	PHBV	PHB4B	PHBHx	PP
Melting temperature (^{o}C)	177	145	150	127	176
Glass transition temperature (^{o}C)	2	-1	-7	-1	-10
Crystallinity (%)	60	56	45	34	50-7-
Tensile strength (MPa)	43	20	26	21	38
Extension to break (%)	5	50	444	400	400

2.5 Biodegradability

Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. Micro-organisms in nature are able to degrade PHAs by using PHA hydrolyses and PHA depolymerases [15]. The activities of these enzymes may vary and depend on the composition of the polymer and the environmental conditions. The degradation rate of a piece of PHB is typically in the order of a few months in anaerobic sewage to years in seawater. UV light can accelerate the degradation of PHAs. PHAs have been proved biocompatible, which means they have no toxic effects in living organisms. Within mammals, the polymer is hydrolysed only slowly. After a 6-month period of implantation in mice, the mass loss was less than 1.6% w⁄w [16]. Figure 2.5 shows the biodegradability of PHB.

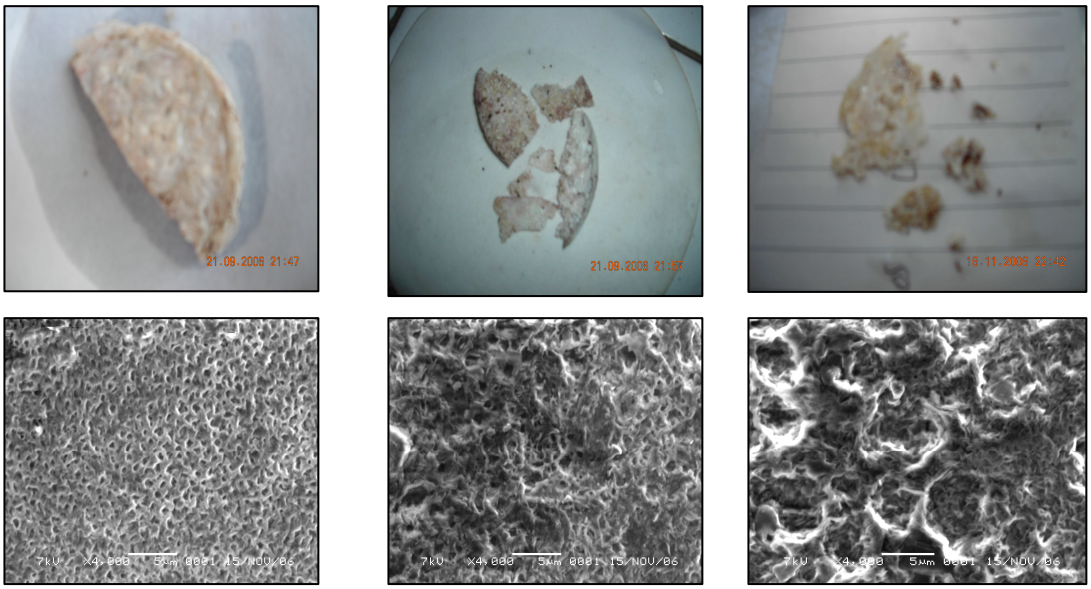

Figure 2.5: The structure of the PHB during (a) One week (b) Two weeks (c) Four weeks

2.7 Application

The majority of expected applications of PHAs are as replacements for petrochemical polymers. The plastics currently used for packaging and coating applications can be replaced partially or entirely by PHAs. The extensive range of physical properties of the PHA family and the extended performance obtainable by chemical modification or blending [17] provide a broad range of potential end-use applications. Applications focus in particular on packaging such as containers and films. In addition, their use as biodegradable personal hygiene articles such as diapers and their packaging have already been described [18].

PHAs have also been processed into toners for printing applications and adhesives for coating applications [19]. Composites of bioplastics are already used in electronic products, like mobile phones [20]. Potential agricultural applications include

encapsulation of seeds, encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities.

PHAs also have numerous medical applications. The main advantage in the medical field is that a biodegradable plastic can be inserted into the human body and does not need to be removed again. PHA has an ideal biocompatibility as it is a product of cell metabolism and also 3-hydroxy butyric acid (the product of degradation) is normally present in blood at concentrations between 0.3 and 1.3 $mmol^{-1}$) . In pure form or as composites with other materials, PHAs are used as sutures, repair patches, orthopedic pins, adhesion barriers, stents, nerve guides and bone marrow scaffolds. An interesting aspect of PHA scaffolds is the fact that the tissue engineered cells can be implanted with the supporting scaffolds. Research shows that PHA materials can be useful in bone healing processes. PHA together with hydroxyapatite (HA) can find an applications as a bioactive and biodegradable composite for applications in hard tissue replacement and regeneration [21].

Polymer implants for targeted drug delivery, an emerging medical application, can be made out of PHAs [22]. However, because of the high level of specifications for plastics used in the human body, not every PHA can be used in medical applications. PHA used in contact with blood has to be free of bacterial endotoxins and consequently there are high requirements for the extraction and purification methods for medical PHAs [23]

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Microorganism

Cupriavidus necator CUG 52238 is used in all experiments. The culture is maintain on nutrient agar broth at 4^{o}C and sub culture every two week.

3.2 Regeneration of the bacteria

The culture is maintained at slant medium. Regeneration is conducted every two weeks. Slant is prepared as the following procedure. Prepare NGY agar medium with the following composition:

Table 3.1 : NGY agar medium composition

Chemicals	Amount (g/L)
Peptone	5
Glucose	10
Yeast extract	3
Beef extract	0.3
Agar	15
Aqueduct	Added until total volume= 1L

The solution is heated in a beaker glass with continuous stirring on laboratory hot plate until the solution comes into boiling. About 10 ml of the hot agar solution is poured into each sterilized test tube. The tube is closed with sterile cotton and wrap in aluminum foil. The tubes are sterilized in autoclave for 30 minutes at 121ºC. The tubes are put in incline position so that the agar will set with inclined surface in the tubes. Let it set for one night in sterile incubator. The bacteria are transferred from the old slant to the new slant in sterile laminar air flow hood with the following procedure:

Firstly, the metal loop is heated until burning red. Then, the old slant containing bacteria to be regenerated is opened. Next, the loop is cooled down by touching it on the agar surface. After that, one loop full of bacteria is scraped and quickly transfer it to the new slant by slightly scratch the agar surface.

The slant is incubated in the sterile incubator at room temperature for about 24 hours until the bacteria seem to grow. Then, keep in the refrigerator at 4ºC for long time maintenance.

3.3 Fermentation for Starter 1

Table 3.2 : Medium for Starter 1 NGY without agar

Chemicals	Amount (g/L)
Peptone	5
Glucose	10
Yeast extract	3
Beef extract	0.3
Aqueduct	Added until total volume= 1L

20 ml of NGY medium is put in 100 ml Erlenmeyer, the flask is closed with sterile cotton then is sterilized in autoclave for 30 minutes at 121ºC. Let it stand in sterile incubator for 24 hours at room temperature. One loop of the bacteria from the slant is